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13. ABSTRACT *(Maximum 200*

We seek to identify the cellular and molecular mechanisms responsible for the regulation of aromatase expression in the breast cancer and surrounding adipose tissue. As previously reported, we completed most of the studies proposed under four specific aims. During the past year, we identified a novel promoter region in the aromatase P450 gene, which regulates aromatase expression in breast tumors and adipose tissue from different body sites. We are currently characterizing the role of this new promoter in the pathophysiology of the breast cancer. From a different perspective, we also concentrated on the mechanisms responsible for intra- and peritumoral accumulation of adipose fibroblasts, which are the major cells that express aromatase in the breast. Malignant cells mediate this accumulation of fibroblasts, *i.e.*, desmoplastic reaction by suppressing the differentiation of these fibroblasts into mature adipocytes. The secretory products of malignant cells and fibroblasts that mediate this inhibitory effect were identified as tumor necrosis factor-α (TNFα) and interleukin (IL)-11. Details are provided in the text.

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Sardar E. Burton 10/9/97

PI - Signature Date

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5. INTRODUCTION

This is the third Annual Report for the Career Development Award entitled "Breast Cancer and Estrogen Biosynthesis in Adipose Tissue" funded by the US Army Medical Research and Material Command Breast Cancer Research Program. This report covers research for the period 9/12/96-9/11/97. As documented in the previous annual report, we have practically completed all the studies that were proposed in this Application. The reviewers of the previous annual report (1995-96) shared our view regarding this conclusion. As a general comment, the reviewers stated that most of the proposed studies have been completed successfully. On the other hand, they made specific comments on some technical issues. In the review, a question was also asked regarding the future aims. In this report, therefore, I will start by summarizing the project and past accomplishments. I will then address the specific technical issues brought about in the past year's review. Next, I will report the data generated during the past year in **6. BODY**. Finally, future aims will be outlined in **6. BODY**.

The long-term goal of this Application is to characterize the molecular and cellular mechanisms responsible for estrogen biosynthesis in adipose tissue surrounding a breast tumor. Since aromatase P450 (P450arom) catalyzes the conversion of C_{19} steroids to estrogens, our studies focus on the expression of P450arom in adipose fibroblasts in relation to a tumor. During preliminary studies, we have detected the highest levels of adipose tissue P450arom transcripts in breast quadrants bearing a tumor compared with tumor-free quadrants. The grant proposal included four specific aims: The **first aim** is to quantify adipose tissue P450arom transcript levels in an additional number of mastectomy specimens at various distances from the tumor using a novel quantitative RT-PCR method. We also proposed to quantify these transcripts in breast samples of women undergoing reduction mammoplasty to ascertain the distribution of aromatase expression. This aim also included determining the ratio of fibroblasts, the aromatase-expressing cell type, to mature adipocytes in these tissue samples. This specific aim has been fully accomplished, and the results have been published as detailed in the previous annual reports. The **second specific aim** is to determine the promoter regions used to express aromatase in adipose tissue samples proximal to a tumor. We initially developed a novel exon-specific quantitative RT-PCR assay. Subsequently, this assay was used to determine the promoter-specific 5'-ends of P450arom transcripts in the tumor tissue as well as in the adipose tissue samples proximal to a tumor. As described in the previous annual report, we found that the two cyclic AMP-responsive promoters (II, I.3) but not the glucocorticoid plus cytokine-inducible promoter (I.4) primarily directed aromatase expression in the breast tumors and the surrounding adipose tissue. **Specific Aim 3** involves characterization of novel 5'-ends of P450arom transcripts in breast cancer tissues. This involves using rapid amplification of cDNA ends, and sequencing to identify potential P450arom promoter regions, which have not yet been described. We previously had the view that the results from studies under specific aim 2 practically ruled out the existence of a yet unidentified tumor-specific promoter, since the sum of the levels of promoter-specific transcripts was roughly equal to total transcript levels as determined by amplifying the common coding region. This, however, was a rough estimate, and since we recently had time for more experiments, we went ahead and performed rapid amplification of 5'-complementary DNA ends (5'-RACE) using total RNA from tumors and

the adipose tissue. We identified a novel untranslated first exon in RNA from four tumor samples and also in adipose tissue samples from the breast, abdomen and buttock. This is strongly suggestive of another promoter region responsible for aromatase expression in the breast cancer and adipose tissue. Finally, the **fourth specific aim** was to determine whether secretory factors of breast cancer cells induce aromatase expression in the surrounding adipose tissue and to characterize such factors. As described in the previous annual reports, interleukin (IL)-6, IL-11, oncostatin M (OSM) and leukemia inhibitory factor (LIF) were found to be expressed in breast cancer tissues, cell lines and adipose fibroblasts. During the past year, we studied the expression of another cytokine that stimulates aromatase activity in adipose fibroblasts, namely, tumor necrosis factor-α (TNF-α) in cancer cell lines T47D and MCF7. We studied the effects of breast cancer cells on the differentiation and proliferation of adipose fibroblasts and found that TNF-α and IL-11 may play critical roles in the desmoplastic reaction, *i.e.*, accumulation of fibroblasts around malignant cells.

6. BODY

I. Firstly, I would like to address the technical issues raised by the reviewers of the previous annual report:

A. We originally proposed to study 40 samples of mastectomy and 40 samples of mammoplasty samples to determine the levels of total and promoter-specific P450arom transcripts in the breast. The results from 18 mastectomy and 9 mammoplasty samples were statistically clear-cut in providing answers for these questions (discussed in the previous annual report). Thus, we will not increase these numbers to 40. On the other hand, we continue to collect more breast tissue samples to determine the distribution of a recently identified promoter-specific transcript (see below) in comparison to already characterized ones.

B. Thus far, we could not find a correlation with the tumor grade and size with the levels of total or promoter-specific transcripts. In future studies, we will continue to test this hypothesis.

C. Although IL-6 and related cytokines are secreted by malignant cells in response to estrogen, the previously reported results under Specific Aim 2 suggested that these cytokines may not be of primary importance for stimulation of intra- and peri-tumoral aromatase expression, because the *in vivo* data demonstrated preferential use of the cAMP inducible promoters I.3 and II but not the cytokine plus glucocorticoid inducible promoter I.4 in breast tumors and the surrounding adipose tissue. Therefore, we will concentrate our future efforts in identifying off-the-shelf substances such as prostanoids as candidate factors for inducing aromatase expression. In the meantime, we identified a critical role of cytokines in the pathophysiology of breast cancer. They seem to mediate the desmoplastic reaction, *i.e.*, formation of the dense capsule made of fibroblasts and collagen in and around the tumor tissue. Our scientific instincts dictated us to concentrate our efforts in identifying the molecular mechanisms responsible for this phenomenon. The details are reported below.

D. In addition to our previously published data, we tested the stimulatory effects of MCF7, SSC202, SSC78, SSC30, MDA-MB157, and MDA-MB415 cell lines on aromatase activity of adipose fibroblasts, as well as the on cytokine production and found similar results to our previously published data. We also obtained similar results using 5 more breast cancer tissues.

II. Using the 5'-RACE procedure, we recently cloned a novel untranslated first exon upstream of the common splice junction of P450arom transcripts (containing the identical common coding region as in other transcripts) from a breast cancer tissue. We designed oligonucleotides to amplify this new exon in 4 breast cancer and surrounding adipose tissue samples. Upon southern hybridization of the amplified products, we estimated that the expression of this new transcript was high in cancer tissues and surrounding fat but relatively lower in breast fat distant to the tumor. These findings should be viewed with

caution, since samples from only 4 mastectomy specimens were tested thus far. In addition, high levels of this novel exon was found in samples of abdominal and buttock fat of cancer-free subjects. From a human genomic library, we recently isolated a 10-kb clone that contains this 100 bp sequence. Once the upstream sequence of the novel exon is sequenced, we will identify promoter and other regulatory elements in this region, determine the transcription start site by primer extension and S1 nuclease assays and prepare deletion constructs for the transcriptional regulation studies. We will also attempt to overlap the new genomic clone with the existing genomic clones of the P450arom gene to estimate the location of the novel first exon and upstream promoter region in reference to the common ATG translation start site. These studies have been previously proposed under Specific Aim 3.

III. The role of cytokines in local estrogen biosynthesis in the breast in relation to a tumor has been a major focus of this Application. Contrary to our expectations, members of the IL-6 cytokine family were not found to be major role-players in stimulating intra- and peri-tumoral aromatase expression in the breast. In the course of our studies, however, we identified an indirect but equally important role of these cytokines and TNFα in regulating local aromatase expression. These cytokines mediate the accumulation of aromatase-expressing fibroblasts around the malignant epithelial cells, *i.e.*, the so-called desmoplastic reaction. In particular, TNFα and IL-11 were found to mediate this effect. The first mechanism appears to be the inhibition of differentiation of adipose fibroblasts (preadipocytes) into mature adipocytes. For example, co-culturing 3T3-L1 murine fibroblasts with T47D and MCF7 cancer cell lines inhibited the differentiation of these cell into adipocytes. Additionally, expression of transcription factors responsible for adipocyte differentiation [*e.g.*, peroxisome proliferator-activated receptor (PPAR)-γ] was found to be inhibited. The addition of neutralizing antibodies against TNFα and IL-11 to the culture medium reversed this inhibitory effect of the cancer cells. On the other hand, labeled thymidine incorporation into 3T3-L1 cells only slightly increased when these cells were co-incubated with cancer cells in the presence of estradiol. These preliminary data suggest that inhibition of fibroblast-to-adipocyte differentiation but not proliferation of these cells is a major tumor effect that mediates the desmoplastic reaction. We are currently determining the major cell type(s) that express TNFα and IL-11 in breast tumors, as well as the mechanisms responsible for the inhibition of various transcription factors involved in adipocyte differentiation.

IV. We also determined that in the adipose tissue of women at various body sites (abdomen, buttock and thigh) primarily use the cytokine plus glucocorticoid inducible promoter I.4 for aromatase expression. (Please see enclosed report.)

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8. APPENDIX

A copy of the representative publication is included in this annual report.

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Alternatively Spliced Transcripts of the Aromatase Cytochrome P450 (*CYP19*) Gene in Adipose Tissue of Women*

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ABSTRACT

Estrogen biosynthesis in adipose tissue has assumed great significance in terms of a number of estrogen-related diseases. The biosynthesis of estrogens from C_{19} steroids is catalyzed by a specific form of cytochrome P450, namely aromatase cytochrome P450 (P450arom; the product of the *CYP19* gene). The human *CYP19* gene comprises nine coding exons, II–X, and its transcripts are expressed in the ovary, placenta, testes, adipose tissue, and brain. Tissue-specific expression of the *CYP19* gene is determined at least in part by the use of tissue-specific promoters, which give rise to transcripts with unique 5′-noncoding termini. Thus, the distal promoter I.1 is responsible for expression uniquely in placenta. On the other hand, the proximal promoter II, which regulates expression via a cAMP-dependent signaling pathway, is responsible for expression in the gonads. Transcripts in breast adipose tissue contain 5′-termini corresponding to expression derived from promoters I.4, II, and I.3, with I.4-specific termini predominating. The latter are derived from promoter I.4, which contains a glucocorticoid response element and an interferon-γ activation site element and is responsible for expression in the presence of glucocorticoids and members of the class I cytokine family. The object of the present study was to determine the distribution of these various transcripts in adipose tissue from abdomen, buttocks, and thighs of women, as this would provide important clues to the factors regulating aromatase expression in these sites. To achieve this, we employed competitive reverse transcription-PCR to amplify unique 5′-ends of each of the transcripts of the *CYP19* gene that are expressed in adipose tissue as well as for the coding region to evaluate total *CYP19* gene (P450arom) transcript levels. We observed that exon I.4-specific transcripts were predominantly present in adipose tissue samples obtained from women regardless of the tissue site or the age of the individual. In these tissues, promoter II- and exon I.3-specific transcripts were present in lower copy numbers. We also demonstrated that in these sites total or exon-specific P450arom transcripts levels increased in direct proportion to advancing age and that transcript levels were the highest in buttocks, followed by thighs, and lowest in the abdomen. These results suggest that in normal human adipose tissue, aromatase expression is mainly under local control by a number of cytokines via paracrine and autocrine mechanisms in the presence of systemic glucocorticoids. (*J Clin Endocrinol Metab* **82:** 70–74, 1997)

ESTROGENS have diverse actions at different body sites of women. Estradiol is produced in the ovarian granulosa cells of premenopausal women, whereas estriol and estradiol are secreted by the placenta (1). In both women and men, estrone is produced in the adipose tissue (2–4), and a substantial fraction of this estrone is further converted to estradiol in the periphery (5). Adipose tissue is the major site of estrogen biosynthesis in postmenopausal women (4, 6). Increased estrogen production in elderly obese women is believed to play a role in the pathogenesis of endometrial cancer (7). Furthermore, estrogen produced by adipose tissue within the breast may act locally to promote the growth of breast carcinomas (8, 9).

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† Supported in part by USPHS Training Grant 5-T32-HD-07190.

Estrogen biosynthesis is catalyzed by an enzyme known as aromatase cytochrome P450 (P450arom; the product of the *CYP19* gene) (10–12). In the human, aromatase expression occurs in a number of human tissues and cell types, including the syncytiotrophoblast of the placenta (13), ovarian granulosa cells (14, 15), testicular Leydig cells (16–18), various sites in the brain (19, 20), as well as adipose tissue (3, 6, 21–23). Hemsell and co-workers first addressed the significance of human adipose tissue as a major source of estrogen production and demonstrated that in both women and men, there is a progressive increase in the efficiency with which circulating androstenedione is converted to estrone with advancing age (2). Subsequently, we have shown that with aging, there is an increase in the specific activity of the aromatase enzyme in adipose stromal cells, and we concluded that this may result in increased estrone production associated with aging (24, 25). Recently, we determined that this age-related increase in aromatase activity in adipose tissue is a result of increased levels of P450arom transcripts in various body sites of women, including buttocks, thighs, and abdomen (26). Moreover, expression was highest in buttocks, with lower levels of expression in thighs and abdomen.

The coding region of the *CYP19* gene spans nine exons beginning with exon II, but 5′-termini (exon I) of aromatase

mini: PII, I.3, and I.4. The amplification products were of the expected size (coding, 194 bp; PII, 305 bp; I.3, 289 bp; I.4, 294 bp). The value in arbitrary units for each P450arom transcript level was obtained from the quantified radioactivity of the amplification products (Fig. 2). These values were normalized to total RNA quantity and to GAPDH amplification products in each sample. With advancing age, there was a progressive increase in total P450arom transcript levels in samples obtained from the buttocks, thighs, and abdomen. This age-dependent increase was statistically significant in the buttocks and thighs (correlation coefficients: Pearson's $R = 0.889$; $P < 0.002$ and $R = 0.817$; $P < 0.05$ respectively; Fig. 2, A and B), whereas this linear trend ($R = 0.704$) did not reach statistical significance ($P < 0.10$) in the abdomen (Fig. 2C). The P450arom transcript levels were highest in the buttocks, followed by the thighs, and lowest in the abdomen. Statistically significant differences were found among these three body sites using parametric repeated measures ANOVA [$p(F) < 0.001$]. This was followed by a Newman-Keuls multiple comparisons test, which showed statistically significant differences between buttocks and thighs ($P < 0.005$) and buttocks and abdomen ($P < 0.005$). Although transcript levels in the thighs were higher than those in the abdomen, this difference did not reach a level of statistical significance.

When the levels of the exon-specific transcripts (PII, I.3, and I.4) were added together, the sum was approximately equal to the measured quantity of total P450arom transcript levels (*i.e.* common coding region) in each case. This indicates that these three species accounted for essentially all of the P450arom transcripts present in the RNA (Fig. 2). I.4-specific transcripts comprised the majority of P450arom mRNA in all adipose tissue samples regardless of age and body site, followed by I.3-specific transcripts, and PII-specific transcripts were present in lowest quantities. A repeated measures parametric ANOVA revealed statistically significant differences among these three types of transcripts at each body site [$p(F) < 0.001$, 0.005, and 0.001 in buttocks, thighs, and abdomen, respectively]. A Newman-Keuls multiple comparisons test revealed statistically significant differences between I.4 and PII ($P < 0.001$, 0.025, and 0.001 in buttocks, thighs, and abdomen, respectively). The differences between I.4 and I.3 were significant ($P < 0.005$) in buttocks and abdomen, but not in thighs. Significant differences were also observed between PII and I.3 in all three body sites ($P < 0.001$, 0.025, and 0.001 in buttocks, thighs, and abdomen, respectively). With advancing age, there was a progressive increase in exon-specific transcripts in samples obtained from the buttocks, thighs, and abdomen (Fig. 2). The age-dependent increase was statistically significant for I.4- and I.3-specific transcripts in samples obtained from buttocks and thighs (Fig. 2, A and B). This linear trend, however, did not reach statistical significance for any exon-specific transcript in the abdomen (Fig. 2C). This may be due to the presence of low levels of P450arom transcripts in abdominal adipose tissue.

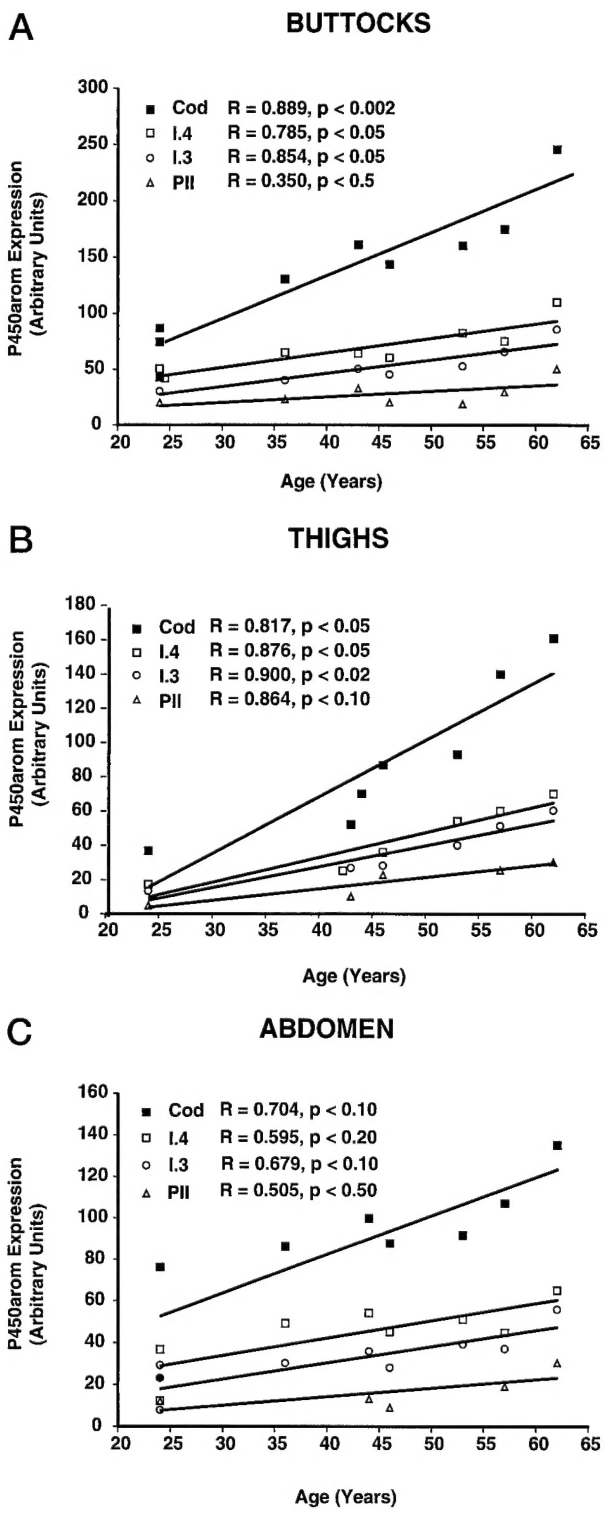

FIG. 2. Amplification of specific 5′-termini of P450arom transcripts in complementary DNA from 1 μg RNA isolated from different body sites of normal women. A, Buttocks; B, thighs; C, abdomen. Data are normalized to GAPDH transcripts. PII, PII-specific transcripts; I.3, I.3-specific transcripts; I.4, I.4-specific transcripts.

Discussion

Recognition of the importance of adipose tissue as a source of estrogens in postmenopausal women came from the pioneering studies of MacDonald and co-workers in the 1970s, who demonstrated that the fractional conversion of androstenedione to estrone in humans increases as a function of

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